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# Histocompatibility bioassays of population structure in marine sponges

Clonal structure in *Verongia longissima* and *Iotrochota birotulata*

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**ABSTRACT:** Clonal population structure in two marine sponges, *Verongia longissima* and *Iotrochota birotulata*, was examined with a self-recognition bioassay. The bioassay consists of grafts of branch segments between conspecific individuals. Results were consistent with the operational properties expected of a precise histocompatibility system. Auto-grafts exhibited acceptance responses; grafts between individuals separated by large distances exhibited rejection responses; individuals were not limited to a single mode of response at one time; and all identity relationships were transitive. Clonal population structure was assessed by examining the relationship between graft response and donor-to-recipient distance, and by actually mapping the distributions of particular clones. Clones of *Iotrochota birotulata* were usually restricted to single coral heads or small patch reefs (1–3 m diameter). For *Verongia longissima*, which can grow directly upon the coral rubble surrounding coral heads and patch reefs, individual clones often occupied larger areas (up to 10 m diameter). The spatial patterns of clonal distributions are readily interpreted as consequences of the particular demographies and habitat specificities of these two species.

THE SPATIAL distribution of asexual lineages or clones is an important component of genetic structure in the populations of many plants and sedentary invertebrates. The preservation of characteristic genotypes by clonal propagation implies that clonal structure can be resolved by any method that assays a sufficiently polymorphic subset of the genome. Polymorphic allozyme and morphological markers have been thus employed to identify clones within natural populations<sup>17,21,31,32,36,37</sup>. However, another class of genetic markers is perhaps ideally suited for the analysis of clonal population structure: those manifested in various self-recognition phenomena.

Self-recognition phenomena, such as self-incompatibility in flowering plants, and histocompatibility in animals, are often under the control of highly polymorphic genetic systems, which effectively provide each sexually derived individual and its clonal descendants in the population with a unique label<sup>2,18,23,26</sup>. The possibility of using self-recognition phenom-

ena as bioassays of clonal population structure has been demonstrated with self-incompatibility in clover and grasses<sup>12–14</sup>, histocompatibility in parthenogenetic vertebrates<sup>1,6,28</sup>, and interclonal aggression in sea anemones<sup>35</sup>. Of potentially much wider application, however, are invertebrate “histocompatibility” systems, which recently have been demonstrated in several major phyla<sup>5,19,20,22,24,25</sup>. If invertebrate histocompatibility systems prove to be generally capable of resolving genetic variation in natural populations, they will then constitute a powerful tool for analysis of population structure in a large and diverse group of organisms.

From what is already known of invertebrate histocompatibility systems and histocompatibility systems in general, it is apparent that not all responses in contacts between conspecific individuals are mediated by precise self-recognition systems<sup>4,7,29</sup>. Furthermore, the resolving power of a self-recognition system, which is presumably a function of inter-clonal genetic differences, may vary among popula-

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tions. It is therefore necessary to test a self-recognition assay in the population in which it is being applied before making a definitive evaluation of its results. Recently we suggested a protocol for testing any self-recognition bioassay of clonal identity<sup>30</sup>. For a true self-recognition phenomenon that distinguishes among clonal genotypes, it is predicted that specific operational properties must characterize the behavior of the phenomenon with respect to genetic relationships: individuals should be capable of responding selectively to specific contacts; interactions between parts of the same individual or between members of the same clone should always elicit self responses; interactions with different clones should always be responded to as non-self; responses should be reproducible; and relationships defined by self responses should be transitive. These operational properties were demonstrated for naturally occurring and experimental histocompatibility contact responses in populations of the reef-building coral *Acropora cervicornis*. The histocompatibility bioassay was then used to describe clonal structure in *Acropora*<sup>30</sup>.

In the present study we have extended the use of histocompatibility analysis of clonal population structure to the most primitive invertebrate phylum, the sponges (Porifera). Certain biological characteristics of sponges, which they share with many other sessile marine invertebrates, make them especially well suited for histocompatibility analysis. Vegetative propagation is widespread in sponges, presumably because of their primitive organization and regenerative capabilities. Sexual reproduction in most sponges involves the production of motile larvae, which settle on suitable substrata, metamorphose into juveniles and become morphologically indistinguishable from vegetatively derived individuals<sup>3</sup>. Clones in sponge populations therefore are initiated by larval recruitment and spread within the habitat by vegetative propagation. Finally, although sponges are characterized by a primitive cellular level of organization, some have been reported to possess a capacity for precise self vs. non-self discrimination that outwardly resembles vertebrate histocompatibility<sup>19,25</sup>.

In this paper we will 1) consider the operational properties exhibited by histocompatibility responses in populations of two marine demospongia, *Verongia longissima* and *Iotrochota birotulata*; 2) examine the spatial distributions of clones defined by histocompatibility assays; 3) discuss clonal structure in populations of these sponges in relation to their utilization of the reef habitat; and 4) evaluate the general effectiveness of the self-recognition



FIGURE 1 *Verongia longissima* at the WFR site of Discovery Bay. The runners of this individual are extended over a coral head.

bioassay approach to population structure analysis.

### Materials and Methods

The field work was conducted at the Discovery Bay Marine Laboratory of the University of the West Indies, Jamaica in March and April 1982. The two species of sponge studied are both common on Caribbean coral reefs at depths less than 20 meters. *Verongia longissima* (Carter) was found in Jamaica on hard substrata in non-cryptic reef locations. The typical growth form was a set of several branches about 1cm in diameter, repent or erect, and originating from an encrusting base attached to either a coral head or loose coral

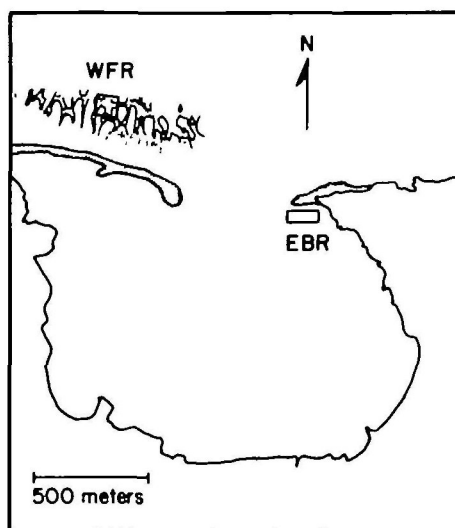


FIGURE 2 Map of Discovery Bay, Jamaica, indicating sites where sponge grafting experiments were conducted. WFR, west fore reef site; EBR, east back reef site.

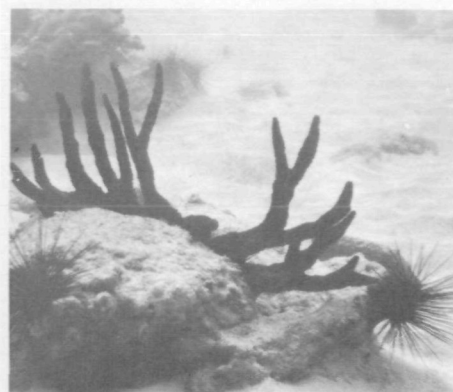


FIGURE 3 *Iotrochota birotulata* at the EBR site. This individual is attached to a non-living coral head, and is isolated from other areas of suitable substrate by sand.

rubble. Some individuals appeared to be actively spreading over the substratum, with their encrusting bases and branches interconnected by runners (Figure 1). Smaller specimens possessed few or no branches. Our study site for this species was on the West Fore Reef (WFR) of Discovery Bay, at a depth of about 15 m (Figure 2). Kaye and Ortiz<sup>25</sup> have previously shown, for a Barbados population of this species, that both "acceptance" and "rejection" responses could be obtained in grafts between individuals.

*Iotrochota birotulata* (Higgin), like *V. longissima*, also is found on hard substratum in open areas, except that it is generally confined to more solid coral heads or patch reefs; it is not found on loose rubble or sand. The typical growth form was a bushy clump of erect or sprawling branches of 1cm to 10cm thickness (Figure 3). This species was studied at two sites in Discovery Bay: on the West Fore Reef, in the same locale where *V. longissima* was studied, and at an East Back Reef (EBR) site where a dense population was found in water 2–10 m deep (Figure 2).

The question of what the term "individual" should refer to when considering colonial animals has long been deliberated<sup>27</sup>, and is especially acute in the case of sponges, where the usual structural criteria for the individual units of colonial organization are inappropriate<sup>10</sup>. Here we will follow the usage of Hartman and Reiswig<sup>16</sup>, which avoids much of this ambiguity, and consider "entire confluent specimens" as individuals.

Grafting experiments were performed in situ with the use of SCUBA. Branch segments 3–10 cm long for *V. longissima* or 10–25 cm long for *I. birotulata* were tied with nylon monofilament to a branch on the same or different individual. Each graft was identified by a plastic tag labeled with a set of punched

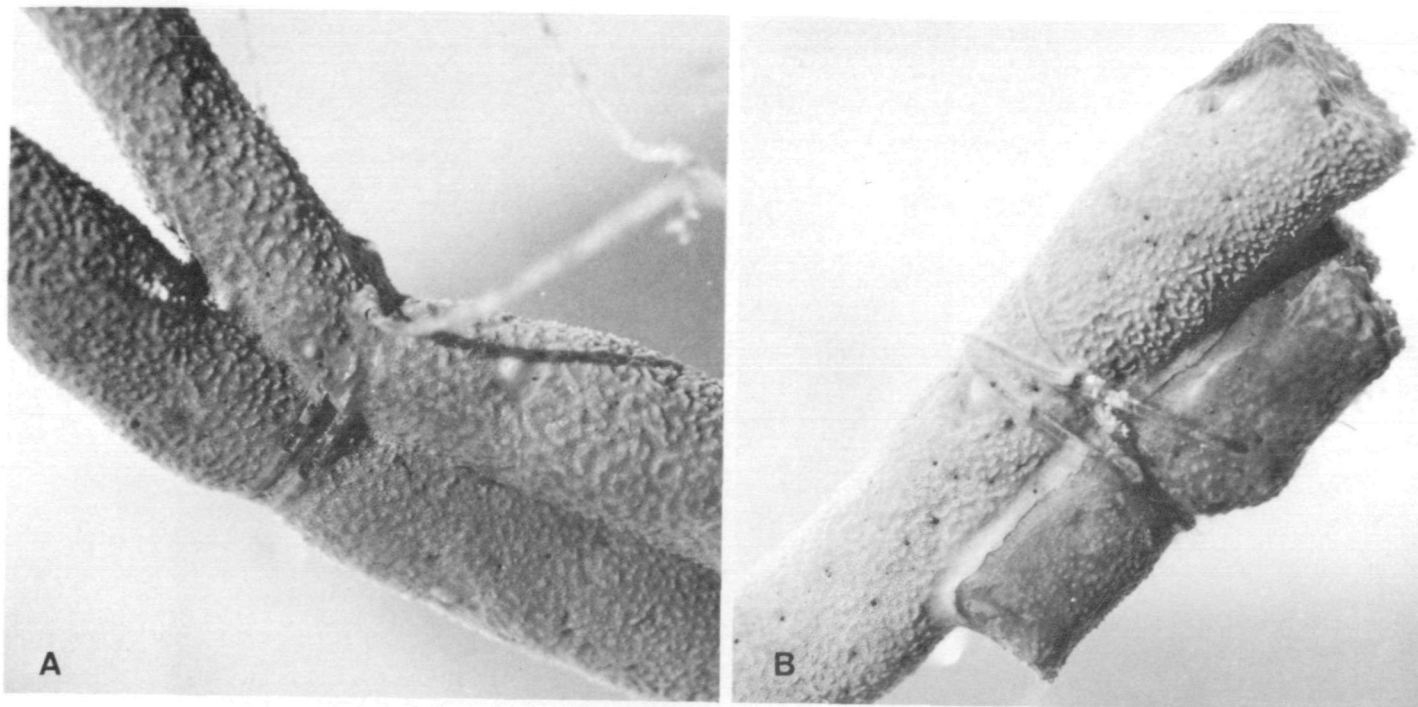


FIGURE 4 Graft responses of *Verongia longissima*. A—acceptance response. B—rejection response.

holes. Scoring of graft responses was essentially “blind” with respect to expected outcomes, because each graft was scored prior to examination of its identification tag. Each graft was scored independently by both JN and JA.

After some preliminary experiments, it was found that grafts of *V. longissima* could be scored within one week, and grafts of *I. birotulata* within 5 days, provided a large enough area of contact ( $>2 \text{ cm}^2$ ) was present at the graft interface. Grafts that had become loose were retied and later rescored. Subsets of grafts for both species were rescored at later times to confirm that initial scorings were reliable.

### Results

Among a total of 181 *V. longissima* grafts performed, 177 (98 percent) were subsequently located and scored after 5–20 days. All grafts were viable, and exhibited either of two characteristic responses—acceptance or rejection. Acceptance responses involved the fusion of the grafted branch segment to the recipient branch, so that an unbroken surface was formed at the graft interface. Rejection responses were initially manifested as a lack of fusion at the graft interface; however, after several weeks a cuticle developed between the donor and recipient branches (Figure 4). Ten grafts were rescored after 7–29 days, and the

results were entirely consistent with the initial scorings. Acceptance and rejection responses, and the histology of graft interfaces have been described by Kaye and Ortiz<sup>25</sup>.

For *I. birotulata*, a total of 285 grafts was performed (26 at the WFR site, and 259 at the EBR site). Among the total, 270 (95 percent) were subsequently located and scored after 5–8 days. Acceptance responses exhibited rapid fusion of the graft and host branches to the extent that their contours merged and no trace of the original graft interface could be detected. A subset of 44 accepting grafts rescored after 9–16 days maintained this appearance. Rejection responses were characterized only by a lack of fusion; cuticle formation, tissue necrosis, or other active manifestations of a rejection were not observed (Figure 5). After 9–16 days, a subset of 78 of these rejecting grafts had all remained unfused, although in every case an adequate area of tissue contact ( $>2 \text{ cm}^2$ ) was present at the graft interface.

### Operational properties

Grafting experiments with *Verongia longissima* and *Iotrochota birotulata* demonstrated the following operational properties:

1) *An individual is not limited to a single mode of response at one time.* Thirteen cases for *V. longissima* and 24 cases for *I. birotulata* were observed where a single

individual responded differentially to grafts received from different donors. This demonstrates the necessary capacity for specific responses to individual donors, rather than a general response to all grafts by an individual sponge.

2) *Self-grafts exhibit acceptance responses.* A total of 13 autografts for *V. longissima* and 37 for *I. birotulata* were made; the procedure involved grafting a branch segment to a new location on the same individual. In all cases the expected acceptance response was observed.

3) *Grafts between individuals separated by large distances exhibit rejection responses.* For the species investigated, which accomplish vegetative dispersal by growth over the substratum, it seemed reasonable to assume that the spatial extent of a clone would be limited. Therefore, if histocompatibility responses were precise, the frequency of graft acceptance between individuals should decrease with the distance between them, and should eventually approach zero. This expected trend was evident for both species (Figure 6). For *V. longissima*, all 68 grafts between individuals greater than 10 m apart exhibited rejection responses. All 105 grafts between *I. birotulata* individuals greater than 2.7 m apart resulted in rejections. These results indicate that interclonal grafts within a population can be accu-

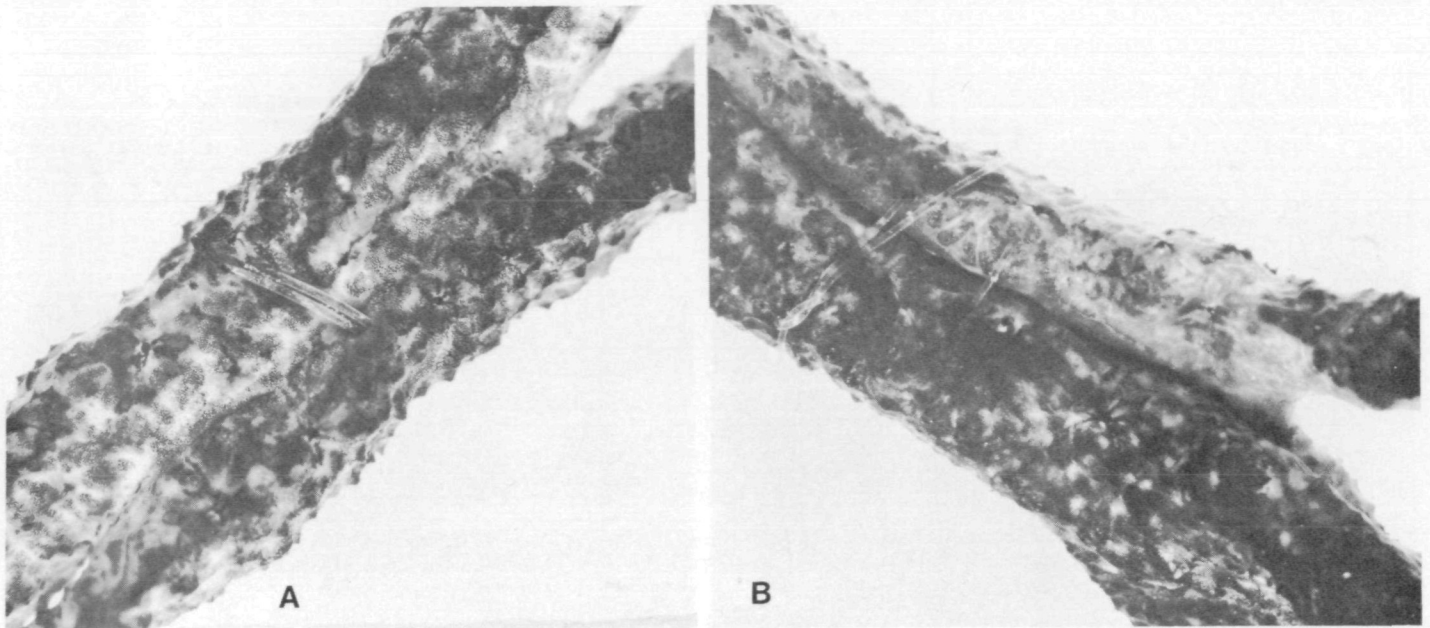


FIGURE 5 Graft responses of *Iotrochota birotulata*. A—acceptance response. B—rejection response.

rately recognized as non-self, and rejected.

4) *Individuals related by graft acceptance respond alike to grafts from other individuals.* If graft acceptance implies genetic identity, putative clonemates should exhibit parallel responses in grafts with other individuals; true identity relationships are necessarily transitive. If graft responses were either influenced by non-genetic factors, or were under the control of an imprecise genetic system, intransitivities could occur. In Table I are presented, for both sponge species, the results of all sets of grafts that defined relationships among three or more individuals. Relationships in which at least one

pair were related by an acceptance response were "predictive," in the sense that one relationship could be predicted from the other two by hypothesizing transitivity. No intransitive relationships were observed.

#### Clonal population structure

For vegetatively propagating organisms, like sponges, the spatial extent of clones ultimately should be limited by their ability to disperse. As described in the previous section, to provide a test of our bioassay's accuracy we assumed the validity of this proposition, and required that grafts between individuals separated by large distances exhibit rejection responses. More explicit assessments of the relationship between donor-to-recipient distance and frequency of graft rejections can be interpreted as descriptions of clonal population structure. A comparison of the two species in our study on this basis (Figure 6), indicates that clones of *V. longissima* tend to be of greater spatial extent than clones of *I. birotulata*.

The number of clones represented in a set of individuals can be determined exactly if all pairwise relationships among those individuals are known. All possible pairwise combinations of grafts between individuals (not including reciprocal grafts) were tested for three sets of sponges. Eight distinct clones of *V. longissima* were identified in a set of 10 individuals within a 1800 m<sup>2</sup> area on the WFR. The two pairs of individuals identified as clonemates also were

the pairs with the smallest donor-to-recipient distances. Five distinct clones of *I. birotulata* were identified in a set of six individuals within a 75 m<sup>2</sup> area on the WFR. In this set, the two individuals identified as clonemates were attached to the same coral head. Each member of a set of seven *I. birotulata* individuals within a 300 m<sup>2</sup> area at the EBR site displayed a unique clonal identity.

Histocompatibility assays also were used to provide direct representations of clonal population structure by actually mapping the spatial distributions of individual clones.

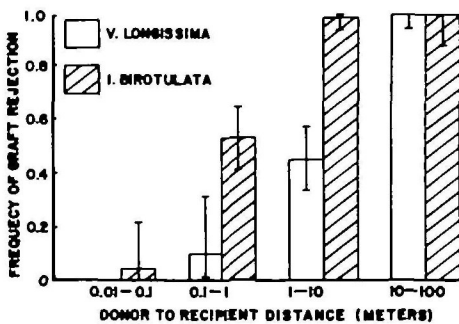


FIGURE 6 Frequency of graft rejection in *Verongia longissima* and *Iotrochota birotulata* for five donor-to-recipient distance classes, with 95 percent confidence intervals.

Table I. Grafting assay defined relationships among sets of three or more individual sponges; values represent number of observations

| Relationship | <i>V. longissima</i> | <i>I. birotulata</i> |
|--------------|----------------------|----------------------|
| Transitive   |                      |                      |
| A = B        |                      |                      |
| A = C        | 10                   | 5                    |
| B = C        |                      |                      |
| A ≠ B        |                      |                      |
| A ≠ C        | 24                   | 12                   |
| B ≠ C        |                      |                      |
| Intransitive |                      |                      |
| A = B        |                      |                      |
| A = C        | 0                    | 0                    |
| B = C        |                      |                      |
| A ≠ B        |                      |                      |
| A ≠ C        | 148                  | 58                   |
| B ≠ C        |                      |                      |



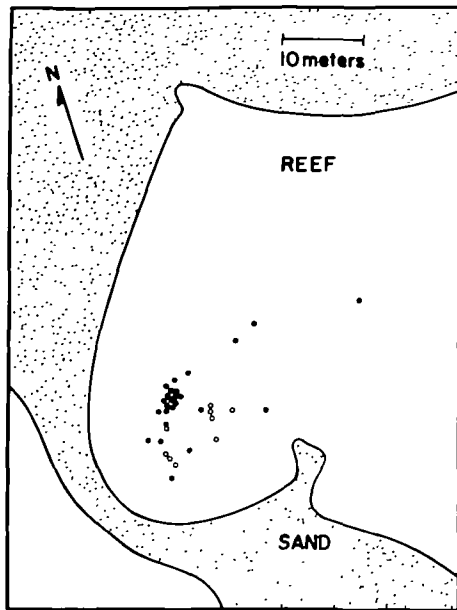


FIGURE 7 Map of a single clone of *Verongia longissima* at the WFR site (Dancing Lady Reef). Solid circles indicate positions of clonemates; open circles, non-clonemates.

Particular clones of *V. longissima* and *I. birotulata* were mapped by selecting individuals in an area, recording their positions, and grafting them with a standard individual. The *V. longissima* clone appeared as a cluster of individuals surrounded by non-clonemates (Figure 7). The map of the *I. birotulata* clone at the EBR site reflected the general pattern of high clonal diversity exhibited by this species; no acceptance responses were found in grafts with the tested individuals. For *V. longissima*, an additional mapping approach was used, which made use of the rapidity of the graft responses and the operational property of transitivity. An area containing a dense concentration of *V. longissima* was selected and grafts among the sponges were made, initially at random. Scoring the initial grafts revealed groups of compatible individuals, which were then extended to include additional individuals and merged into larger groups by the application of further grafting comparisons. Many relationships were independently verified by multiple graft comparisons. A total of 44 grafts was made within this set, which unambiguously assigned each of the 35 grafted individuals to one of three clones. An additional 53 individuals were located in this area; these were tentatively assigned to one of the three clones on the basis of their proximity to individuals of known clonal identity. The resulting multi-clone map is shown in Figure 8. Characteristic of all four

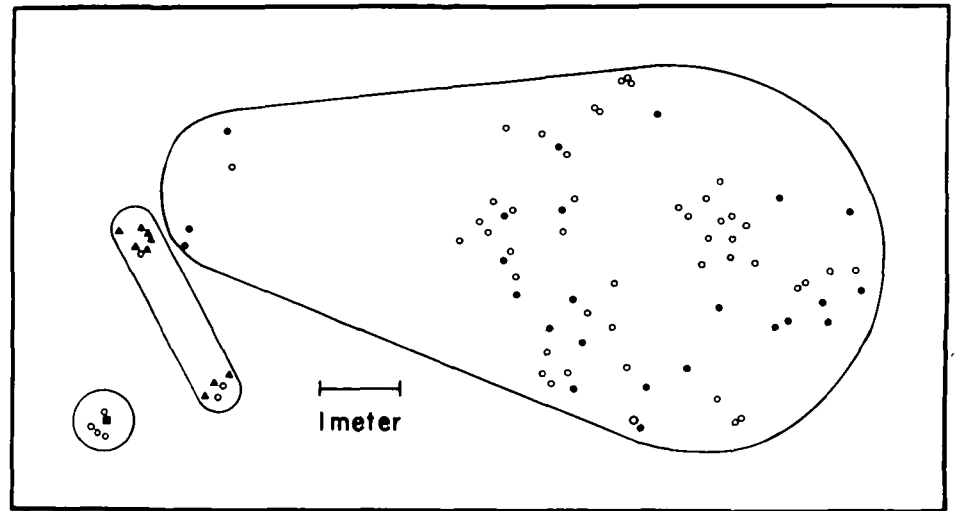


FIGURE 8 Map of *Verongia longissima* at the WFR site (LTS Reef). Solid circles, triangles, and square indicate the specific clonal identities determined for individuals at those positions; open circles indicate individuals located in the mapped area, but not used in graft comparisons.

mapped *V. longissima* clones is the homogeneity of their distributions; boundaries could be defined that included all the members of a specific clone but did not include any members of other clones.

#### Habitat correlation

There were no obvious features of the habitat that corresponded with the distributions of *V. longissima* clones. However, an interesting relationship between clonal distribution and habitat structure is suggested by the *I. birotulata* grafting data. Clones appeared to be restricted to single coral heads or solid patch reefs, the discrete "patches" of the habitat utilized by this species. Grafts were made between individuals on separate habitat patches, and between individuals occurring within the same patch. The graft responses and donor-to-recipient distances for these experiments are shown graphically in Figure 9. There were only three examples, out of 127 graft comparisons between individuals on separate habitat patches, of a clone being represented in two rather than one habitat patch. However, *I. birotulata* graft responses also showed a parallel dependence on distance alone, both for within-patch and between-patch comparisons. There were no apparent differences in *I. birotulata* clonal population structure between the WFR and EBR sites.

#### Discussion

Self-recognition phenomena in lower animals, and histocompatibility in particular, may provide a general means of detecting ex-

tensive genetic polymorphisms that can serve as clone-specific markers. The possibility of such an approach had not been considered until recently. It generally had been believed that a system for precise self vs. non-self discrimination was a special feature of vertebrate evolution. The complexity of the vertebrate immune system, with its architecture of specialized cells, tissues and organs, seemed to preclude a pre-vertebrate origin<sup>8,9,11</sup>. Results of early transplantation studies generally upheld this view; lower invertebrate hosts appeared to accept any physiologically compatible tissue, even across species boundaries<sup>4,29</sup>. However, a search for the pre-immunocompetent stage of metazoan evolution among living representatives of the invertebrates recently led Hildemann and co-workers<sup>19,20</sup> to conclude that histocompatibility systems, characterized by precision and immunological memory, could be found in even the lowest multicellular animals.

To reconcile the apparent contradiction between the early findings of indiscriminant compatibility, and some more recent findings of precise allogeneic incompatibility, it must be concluded that either the choice of organisms has been critical in these studies, or alternatively, differences in technique and methodology have played a larger role than is generally suspected. Of the latter, it may be significant that precise allogeneic graft discrimination has been consistently found when whole sponges, or segments with their outer pinacodermal covering still intact, are placed in apposition<sup>19,25</sup> (and this study); and where lack of discrimination, or ambiguous results

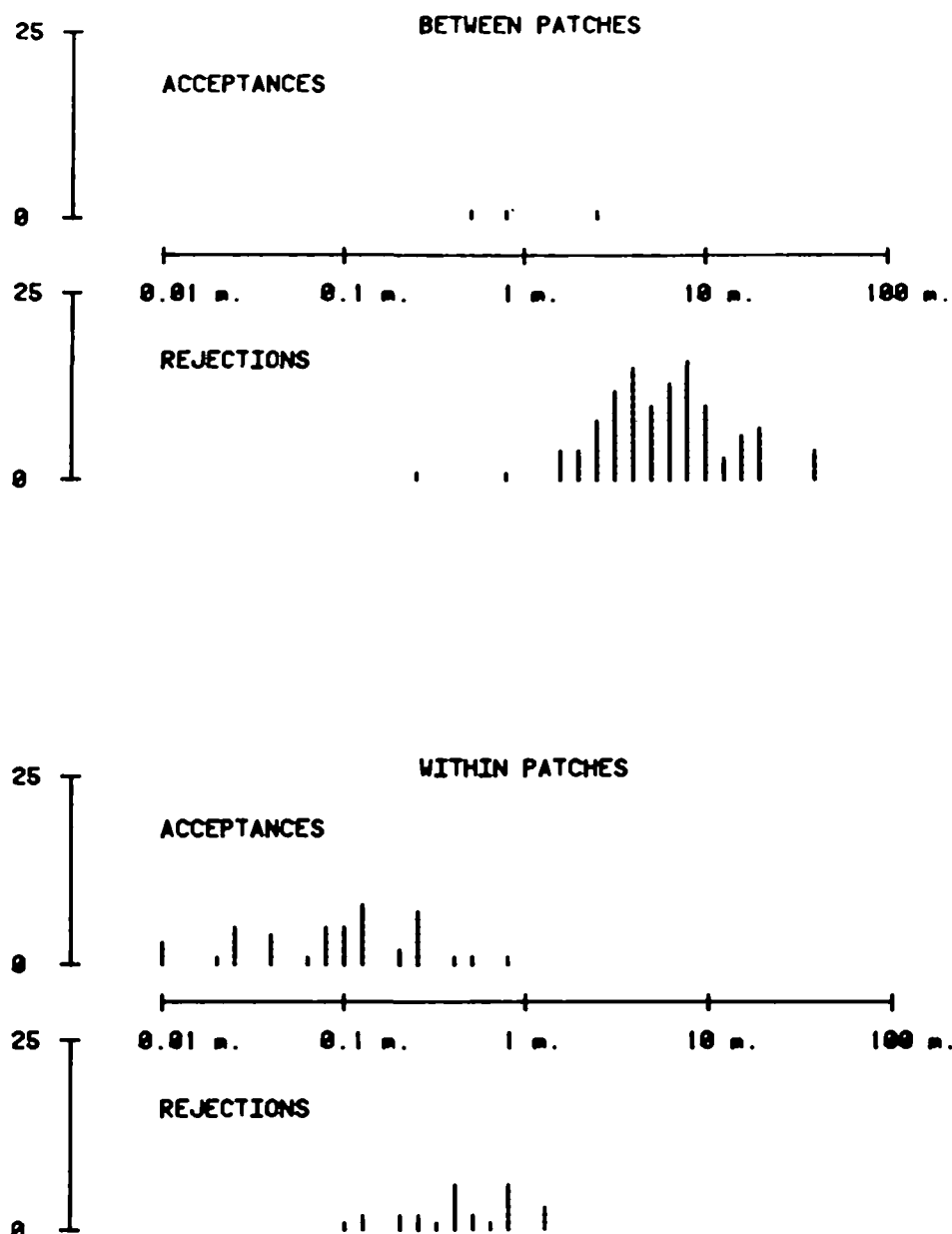


FIGURE 9 Graft responses and donor-to-recipient distances (logarithmic scale) for grafts between *Iotrochota birotulata* from different patches of suitable substrate, and between individuals from the same patches.

have been obtained, the technique has involved the subdermal insertion of a "core" excised from the donor, through an incision made in the host<sup>7,29</sup>. The potential importance of technique became apparent in our study with *Iotrochota birotulata*. The branch segments we ultimately used as grafts were as large as many of the individuals in the population, and so the distinction between "donor" and "recipient" has simply been used as a convenience to describe the final location of a pair of grafted individuals. In preliminary experiments, where small explants scraped free of

pinacoderm were inserted subdermally into hosts of the same species, we could not observe any distinct responses that would distinguish allografts from autografts (unpublished observations). For *I. birotulata*, graft rejections could only be detected as a passive lack of fusion between apposed pinacoderms.

By defining the behavior of a self-recognition phenomenon in terms of operational properties, experimental methods are included as part of the phenomenon. This convention can be used to address the suitability of the phenomenon as a workable bioassay, which

requires not only the existence of the biological self-recognition system, but also a method of extracting information from it accurately. The approach is limited only by the availability of a set of known genetic relationships to serve as controls, and the assumption that they are representative of the unknown relationships being determined. We have introduced one method of testing the assumption that histocompatibility responses in the populations we have studied accurately reflect clonal relationships. By examining assay-defined relationships among sets of three or more individuals, identity relationships can be characterized as either transitive or intransitive. Because in our study identity relationships were always transitive, we conclude that: 1) grafting responses were reproducible (alternative outcomes would have produced intransitivities), and 2) relationships defined by graft compatibility fit a model in which complete matching of histocompatibility genotypes is required for a graft acceptance. Thus, there was no evidence for a threshold model in which graft acceptances could occur when histocompatibility genotypes were similar, but not identical. This is an important result, because a threshold system of histocompatibility has been demonstrated for at least one invertebrate, the colonial ascidian, *Botryllus schlosseri*<sup>34</sup>. In the *Botryllus* system, one locus with many alleles determines compatibility. If two individuals share either one or both alleles their tissues are compatible (a one-allele threshold). Thus, all parent-offspring and many sibling relationships are compatible. Intransitive compatibilities would be observed among *Botryllus* colonies with the allelic compositions AB, BC, and CD.

The population genetic structures evident from spatial distributions of histocompatible individuals of *V. longissima* and *I. birotulata* can be readily explained in terms of clonal propagation. This interpretation is consistent with the results of our experimental grafting studies, and with our observations of individuals in the process of extending branches or runners across the substrate. However, sexual reproduction potentially could have a similar effect on genetic population structure, if gamete and larval dispersal were so limited as to almost completely restrict gene flow within a population<sup>33,38</sup>. Inbred patches of closely related individuals, monomorphic for the genetic determinants of histocompatibility, would appear as single clones. This caveat is shared by all methods, including morphological and electrophoretic methods, that examine a subset of the genome to characterize clones. We have assumed that gene flow within the populations studied was sufficient to prevent extreme inbreeding effects.

The results of the present study suggest one way in which microgeographic habitat variation may influence clonal distributions in populations of *Iotrochota birotulata*. The vegetative extension of *I. birotulata* is ultimately constrained by the spatial limits of coral heads and solid patch reefs upon which it can attach. Small distances (up to approximately 1 m) across stretches of unsuitable substratum can be traversed by extended branches (cover photo), while greater distances over sand or loose coral rubble probably act as barriers to vegetative expansion. The distributions of *I. birotulata* clones appear to reflect this limitation on vegetative propagation. Clones were restricted to single coral heads or patch reefs, except for two instances in which the distance between patches occupied by a clone were within the range an extended branch could traverse. In contrast with *I. birotulata*, *V. longissima* can utilize loose coral rubble as a substratum for attachment. The more extensive clonal distributions of this species did not appear to have been molded by any recognizable features of the habitat. In a previous study of clonal structure in populations of the reef-building coral, *Acropora cervicornis*, simple models incorporating empirically estimated rates of vegetative propagation, mortality, and sexual recruitment could account for both qualitative and quantitative features of the observed clonal distributions<sup>30</sup>. These examples suggest that a detailed consideration of basic demographic processes may provide immediate explanations for observed patterns of clonal structure. The use of histocompatibility and other self-recognition phenomena to determine clonal structure in populations of lower animals can provide many opportunities for analyses of these systems.

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